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Vanessa L. Ford
Biotechnology Patent Examiner
Office: CM1 8A16
Mailbox: CM1 8E12
Phone: 703.308.4735
Art Unit: 1645

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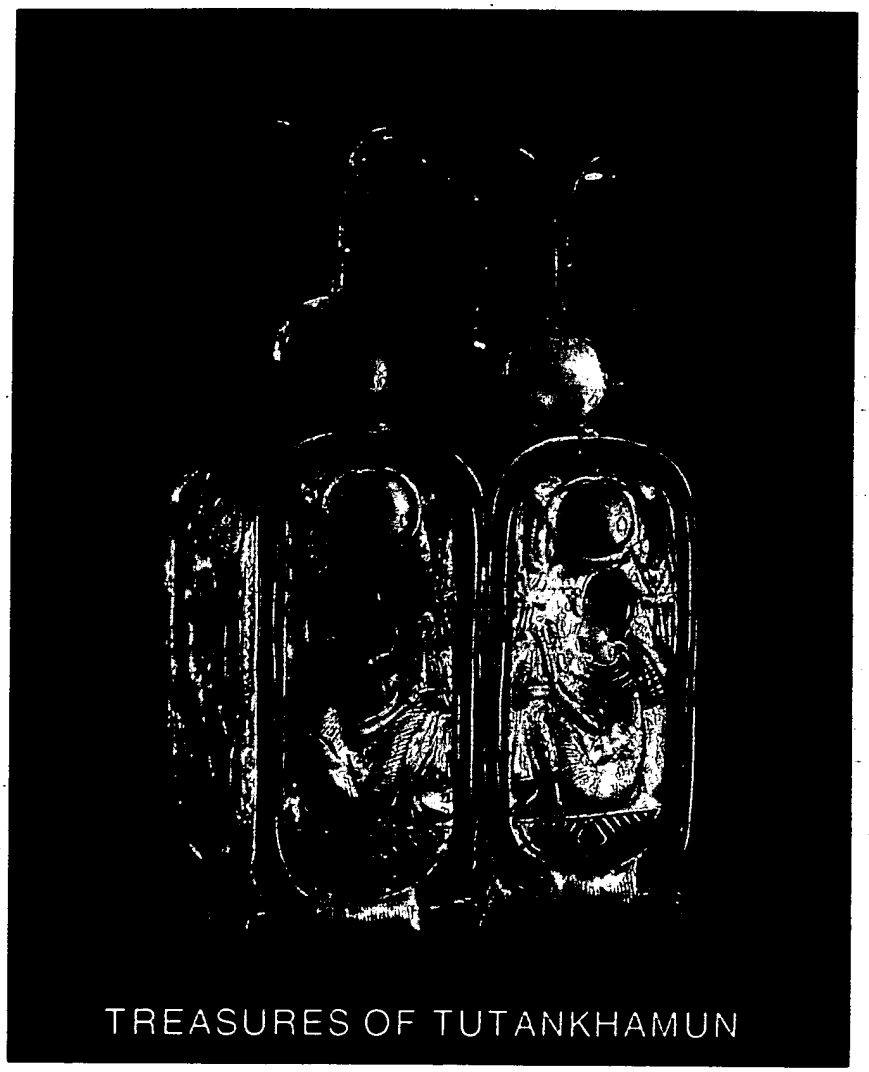

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IMMUNOLOGICAL AND PARASITOLOGICAL STUDIES OF *CRYPTOSPORIDIUM MURIS*, TYZZER (1907)

By

NAZEM M. ABDEL-MAKSOUD[1], AHMED K. DYAB[2] AND MOSTAFA A. SHATAT[3]

Department of Zoology, Faculty of Science, South Valley University[1] and Departments of Parasitology, Faculty of Medicine, Assiut University[2] and Al-Azhar Unviersity[3] (Assiut), Egypt

ABSTRACT

Oocysts of *C. muris* and the events of excystation using 0.5% sodium hypochlorite as excystation medium were described with light microscope. The response of the immunocompetent BALB/c mice against infection was studied using sera of orally infected mice at different periods postinoculation by indirect immunofluorescence antibody test using 1:150 FITC conjugated rabbit serum antimouse polyclonal IgG. From the patterns of IFAT, it was suggested that the dominant antigen in *C. muris* was restricted to the apical complex of the sporozoites. Such antigen may play a role in the invasion of the host cell. Future analysis of such receptor molecules might constitute prime candidates as immunogens for a vaccine, the efficiency of which might cause inhibition of parasite invasion.

INTRODUCTION

Cyrptosporidiosis is an infectious disease caused by a coccidian parasite belonging to the genus *Cryptosporidium* (Uga et al., 1989). The protozoan was first detected in the gastric glands of the mouse by Tyzzer (1907 and 1910) and has been known as a cause of parasitic zoonosis since the first human case

was reported (Nine et al., 1976). Later, the parasite has attracted greater attention as it causes severe diarrhoea in AIDS patients. Two named species of the protozoan parasite *Cryptosporidium* were detected in man, the highly prevalent was *C. parvum* and the less prevalent *C. muris* (Garone et al., 1986. Probably, Clarke (1895) was the first to observe *C. muris.* He termed them "swarm spores" lying free upon the gastric epithelium of mice. Tyzzer (1907-1910) reported that infected mice grew less rapidly. *C. muris* lacked host specificity and could be experimentally transmitted to mice, rats, guinea pigs, rabbits, dogs and cats (Iseki, 1988). Garone et al. (1986) reported the first case of human infection with *C. muris* in the stomach of a homosexual male patient with AIDS who developed symptoms suggestive of gastric outlet obstruction during the course of severe chronic diarrhoea due to cryptosporidiosis. *C. muris* in cattle has been associated with long-standing gastric (abomasal) cryptosporidiosis and reduced weight gain (Anderson, 1987). Animals infected with *C. muris* had significantly elevated plasma pepsinogen levels and a significantly lower weight increment than healthy animals of comparable age (Anderson, 1989). Infected animals exhibited clinical signs that include chronic loose stools, reduced appetite, weight loss and lethargy (Anderson, 1991). *C. muris* has been isolated from different animals (Upton and Current, 1985) calves, (Iseki, 1986) rat, (Anderson, 1989) cattle and camel (Anderson, 1991) with apparent differences in size that leads to speculation about multiple strains and even multiple subspecies. In Egypt, many reports on human cryptosporidiosis have been published as Azab et al. (1985), El-Saifi et al. (1985); Abou El-Maged and Abou-Shady, (1986); Nour et al. (1988); Boghdadi, (1990); Awadalla et al. (1995); Aboul Assad and Yasseen (1996); Youssef et al. (1998). However, complete illustration of the parasite was not given. Hence, the present work was proposed for the detailed description of oocysts of *C. muris*, the process of their excystation and the host response using the serum antibody IgG by indirect immunofluoresence antibody technique (IFAT).

MATERIAL AND METHODS

The large type *Cryptosporidium* used was previously isolated from *Rattus norvegicus* by senior author and passage in six weeks old specific pathogen free (SPF) immuocompetent male BALB/c mice. Oocysts were

concentrated from the faeces of infected SPF mice by the centrifugal floatation using sugar solution (sp. gr. 1.2), washed with distilled water, resuspended in an aqueous solution of potassium dichromate ($K_2Cr_2O_7$) 2% stored at 4°C for less than three months. Two groups (6 mice each) of six weeks old adult 25 gram male immunocompetent BALB/c mice were used. The first group was orally inoculated with $5x10^5$ highly ppurfied oocysts of *C. muris* (Uni et al., 1987) and had free access to food and water, the other group was kept without infection and taken as a control group. To determine the excystation process, oocysts were concentrated by floatation in sheather's sugar solution, washed four times by centrifugation in phosphate buffered saline (PBS) and subsequently incubated in 0.5% sodium hypochlorite in PBS of pH 7.4 at 37°C for 15 minutes and then examined by light microscope and Nomarski interference phase contract microscope. Events of excystation were photographed with NIC microscopy.

Immunofluorescence: Excysted sporozoites were washed free of excystation solution in PBS and bovine serum albumin. HCL coated slides were prepared using 0.1 N Hcl for thirty seconds. The slides were dried and a mixture of oocysts and sporozoites antigens was poured on the slides which were air dried for IFAT use. Serum samples diluted at 1:65536 and 1:150 diluted rabbit antimouse polyclonal IgG conjugated to FITC was added and incubated for thirty minutes at 37°C. Slides were washed in PBS, mounted using a mixture of glycerol and PBS (1:1) and examined by a fluorescence microscope.

RESULTS

C. muris ocysts were fully sporulated in fresh faeces, ellipsoidal (Fig. 1,2); measuring 7.5 - 9.8 x 5.5-7 µm (average 8.4 x 6.3). Wall smooth, colourless, composed of a single thin layer ~0.75 um thick. A faint longitudinal suture extended from narrow pole about 1/3 of oocyst length. Micropyle and polar granules absent. Oocyst residuum represented by a single layer of residual granules 0.2-1.8 µm in diameter. A large spherical or ovoid membrane bound globule 4-5.5 x 3.8-5 µm (average 4.7 x 4.6) present within oocyst enclosed by residiuum without constant position inside oocyst. Sporozoites four vermiform structures measuring 9-11.5 x 1.1.6 (average

10.25 x 1.3) in situ, lying lengthwise and parallel along one side of oocyst, (in excystation conditions, sporozoites occupy whole body) (Fig. 3,4), much longer than oocyst, bent U or sometimes C-shaped over surface of globule (Fig. 1,2). Anterior end of which lied adjacent to narrow pole of oocyst where suture located. Posterior ends reflected and extended around residuum to about half of oocyst length. Sporozoite refractile bodies absent, nucleus posteriorly. Within twenty minutes after addition of the excystation solution, the oocyst wall separated at terminal suture (Fig. 3,4,5) and sporozoites get out through suture. No constant sequence for excystation; in some cases sporozoites began to exit then followed by residuum while in another cases v.v. The residuum broke into small granules just before exit and sometimes broke in excystation solution 1-3 minutes after excystation. Excystation was indicated by the appearance of a shallow indentation at suture site at narrow pole of oocyst. Indentation, sometimes deeper and longer. Sporozoites left anterior end first and oocasionally one or more sporozoites left oocyst simultaneously. In oocysts with narrow opening, sporozoites constricted as they left oocyst (Fig. 3,4). Oocysts treated with sodium hypochlorite had a slightly thinner oocyst wall than non treated ones, with neither difference in size nor shape.

Immunofluorescence: When serum samples from uninfected mice were used as the primary antibody, no fluorescence was observed. Serum samples from mice two weeks postinoculation (PI) showed weak flourescence and conjugated with the sporozoite and the apical complex with antibody titer 1:4. IFAT for sera of mice four weeks PI showed high reactivity with the sporozoite and apical complex (Fig.6) with higher antibody titer 1:64 where fluorescence was observed to be on the apical complex (Fig. 7). All tested sera from mice six weeks PI exhibited a strong conjugation with the antigens in the sporozoites and apical complex with antibody titer 1:256; where only the apical complex of the sporozoite showed fluorescence. Refractile granules vary in number (2 - 4).

DISCUSSION

Eimeria and *Isospora*, are characterized by oocysts possessing inner sporocysts, each of which contains infective sporozoites. Many *Eimeria* and

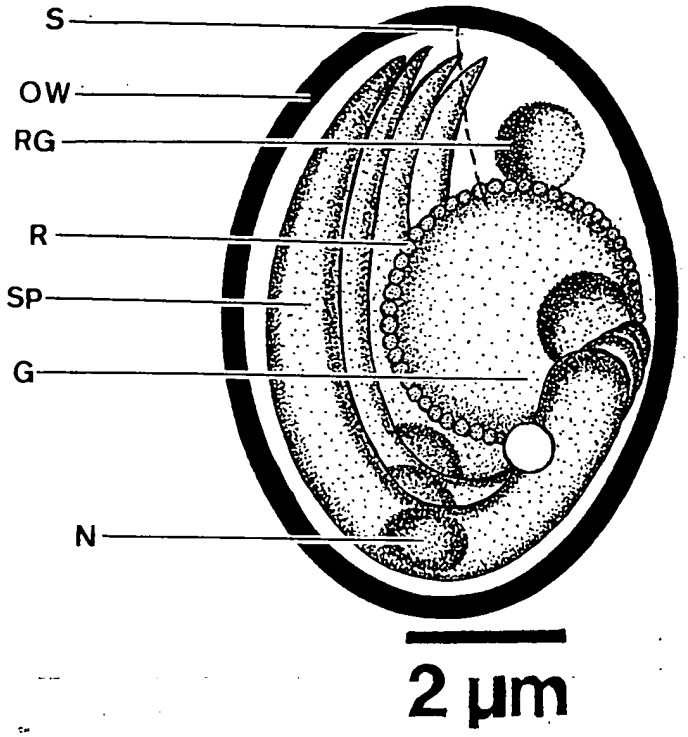

Fig. (1): Line drawing of *C. muris* oocyst strain RN66. Suture, OW: oocyst wall, RG:refractile granule, R: residuum, SR: sporozoite, G: globule, N: nucleus.

Isospora species have thinning of the oocyst wall at one pole, termed micropyle. The presence of a suture in *Cryptosporidium* species indicates its close phylogenetic relationship to the sarcocystids of family Calyptosporidae *(Goussia, Calyptospora)* (Bamforth et al., 1985). The suture in *Cryptosporidium* species is located in the oocyst wall whereas the suture in sarcocystids and calyptosporids lies within the sporocyst walls (Uni et al., 1987). In this context; no uniting membrane was retained between the parasite and the host cell. Besides coccidian oocyst is characterized by the presence of stieda body and a large refractile globule at the anterior end of the sporozoite (Upton and Current 1985). In the present study, neither stieda body in the oocyst nor refractile globule in the sporozoite was seen. *C. muris*

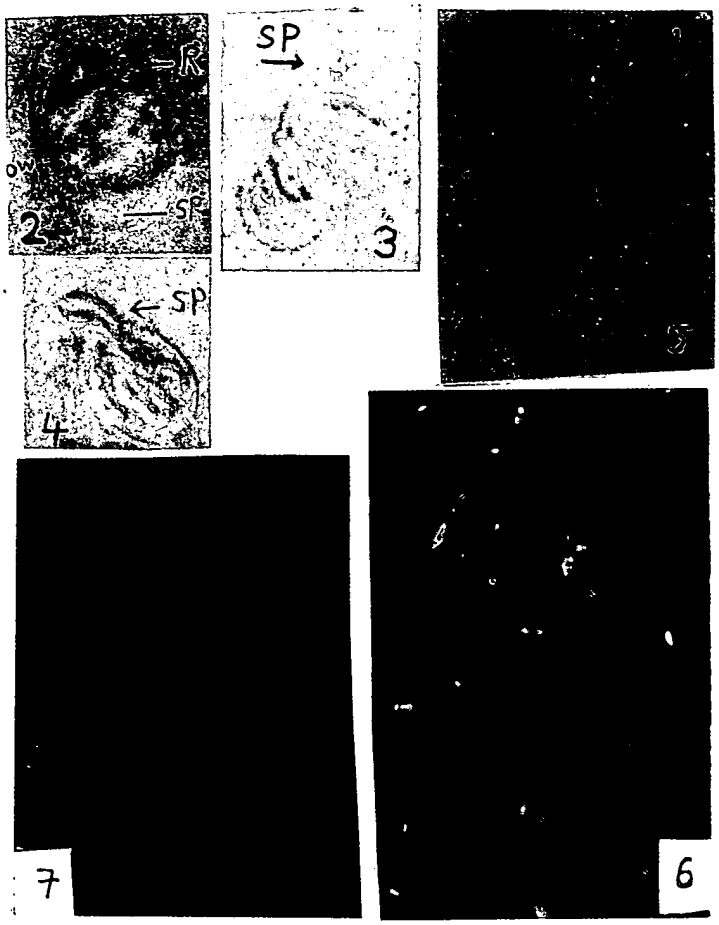

Fig. (2): Oocyst of *C. muris* RN66.

Fig. (3): Excysted oocyst of *C. muris* RN 66 shows constriction of sporozoites during their exit through a narrow suture.

Fig. (4): Oocyst of *C. muris* RN66 shows exit of sporozoites through a large suture.

Fig. (5): Oocyst of *C. muris* after excystation.

Fig. (6): Immunofluorescence on sporozoites under 40 x magnification performed with sera orally infected mice. Polyclonal IgG bind to sporozoite antigen at low antibody titer.

Fig. (7): Immunofluorescence on apical complex of sporozoites under 40 x magnification performed with sera orally infected mice, four and six weeks PI. Polyclonal IgG bind to apical complex of sporozoite antigen at higher antibody titer.

contains no residuum (Upton and Current, 1985). The present study confirmed the presence of a thin residuum composed of a single layer of small unequal granules (Fig. 1,2). Upton and Current (1985) may have been describing a new strain as their parasite was isolated from calves. Cryptospoidiidae was revised with *C. muris* as a type species (Bamforth et al., 1985) occurring in the small intestine of a laboratory mouse, oocysts ovoid or spherical, 4-5 by 3 µm, with a small, knob-like attachment organelle, sporozoites slender, bow-or boomerang-shaped, 5-6 µm long. In the present study, a small amount of both the gastric and intestinal mucosa from infected mice was scraped off and examined, only the gastric mucosa were filled with different stages of *C. muris*, but never in the small intestine . So the amended description is suggested to consider *C. muris* belonging to family Cryptosporidiidae.

Oocysts fully sporulated in fresh faeces, spherical or ellipsoidal in shape, large 7.5-9.8 x 5.5-7 µm (average 8.4 x 6.3). Sporozoites with no visible flagella. Micropyle absent, residuum present, sometimes absent. Stieda body absent. Sporozoite lacked large refractile globule. Globule large and enclosed by residuum, polar one absent. Suture present at narrow pole. Sporozoites four vermiform in shape measuring 9-11.5 x 1-1.6 (average 10.25 x 1.3 Um). Refractile granules present. Development is complete in one host.

The protective immune response in animals against coccidial infection is directed against the sporozoite stage, but it is generally accepted that the asexual stages produce the strongest stimulus for development of immunity (Angstine and Danfothe, 1987). However, because of the antigens of accessibly, the sporozoites have been more vigorously studied. Antigens are present on the sporozoite surface and some of these molecules may be involved in recognition and penetration of host cells. From the present data, it was evident that the fluorescence was associated with the sporozoites especially the apical complex. The patterns of fluorescence were similar except the antibody titer. Therefore, the apical complex of the sporozoite contains a protein (antigen), dominant antigen that causes circulation of antibodies in the sera. On the other hand, the present study confirmed the presence of circulating antibodies against *C. muris* in sera of

immunologically normal individuals recovered from cryptosporidiosis. The presence of a dominant antigen on the apical complex of the sporozoite required analysis by molecular biology.

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